Hydrophobic C-Terminal Amino Acids in the β-Subunit Are Involved in Assembly with the α-Subunit of Na,K-ATPase†

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ABSTRACT: To define the structural basis of oligomerization for the α- and β-subunits of Na,K-ATPase, we have attempted to identify the amino acids in the C-terminus of the β-subunit that are involved in subunit assembly. We predicted that the last 10 amino acids form a β-strand-like structure exposing on one side a hydrophilic and on the other side a continuous hydrophobic domain. The relative importance of the two domains in assembly was probed by introducing point mutations in either domain of *Xenopus* β_3-subunits and by testing the ability of these mutants to stabilize newly synthesized α-subunits expressed in *Xenopus* oocytes and to form functional α–β complexes at the plasma membrane. All single and double mutants with changes at R268 and/or K272 to either uncharged or negatively charged amino acids associated with coexpressed α-subunits and increased the number of ouabain binding sites and Rb uptake into oocytes. On the other hand, mutations affecting the hydrophobic amino acids influenced the assembly efficiency with α-subunits to a variable extent. The single mutants V269N and I275N did not influence and the mutant V273N slightly affected the assembly process. On the other hand, the cellular accumulation of α-subunits and the expression of functional Na,K pumps was considerably reduced with the mutant F271N and totally abolished with the double mutant V269N/F271N. Finally, replacement of V269 *and* F271 or V273 *and* I275 with the less hydrophobic alanine also significantly decreased subunit assembly, which was no longer detectable after replacement of all four amino acids. These data suggest that hydrophobic rather than hydrophilic amino acids in an amphipathic C-terminal β-strand of the β-subunit play a most pronounced role in assembly with α-subunit.

The minimal functional unit of the Na,K-ATPase or Na,K pump expressed at the plasma membrane of animal cells consists of a catalytic α-subunit and a glycoproteinic β-subunit. The α-subunit carries all functional domains for cation, ATP, and phosphate binding. On the other hand, the β-subunit is needed for the stabilization and intracellular transport of the newly synthesized α-subunit [for review see Geering, (1991)]. Recent findings suggest that the β-subunit is also involved in a fine control of the activity of Na,K-ATPase expressed at the cell surface (Eakle et al., 1992; Jaisser et al., 1992; Lutsenko & Kaplan, 1992; Schmalzing et al., 1992). Concomitant synthesis of the α- and β-subunits is not a prerequisite for assembly (Ackermann & Geering, 1992), but oligomerization of the two subunits occurs at the level of the endoplasmic reticulum (ER) (Fambrough & Bayne, 1983; Ackermann & Gerring, 1990). Oligomerization provokes a conformational change that renders the α-subunit resistant to trypsin treatment (Geering et al., 1989; Noguchi et al., 1990) or to cellular degradation (Ackermann & Geering, 1990) and allows the α–β complex to exit from the ER (Jaunin et al., 1992).

The structural domain(s) implicated in the interaction(s) between the α- and β-subunits are still poorly defined. The fact that β-subunits from different species (Horowitz et al., 1990; Schmalzing et al., 1992; Takeyasu et al., 1987) or even from different P-type ATPases (Horisberger et al., 1991) are able to form functional hybrid Na,K-ATPase complexes indicates that the structural determinants for assembly have been conserved during evolution. In contrast to the α-subunit, which is currently believed to span the membrane 8–10 times and exposes most of its mass to the cytoplasmic face, the β-subunit has a short cytoplasmic N-terminal domain, one transmembrane domain with the bulk of the molecule being on the extracytoplasmic face. A high degree of sequence identity between β-isoforms, β-subunits from different species (Lingrel et al., 1990), and β-subunits from Na,K- and H,K-ATPase (Reuben et al., 1990) exists in the N-terminal cytoplasmic and the transmembrane domains. Potentially, these domains could be important for the conserved function of subunit assembly. However, Renaud et al. (1991) have recently reported that cytoplasmic and transmembrane deletion mutants are all able to normally assemble with α-subunits as long as they are capable of ER membrane insertion. Together with the observation that deletions of 11 amino acids from the extracytoplasmic domain abolish subunit assembly (Renaud & Fambrough, 1991), these results suggest that the structural domains involved in assembly are primarily located in the ectodomain of the β-subunit.

Sequence analysis reveals a certain degree of diversity in the 11 most C-terminal amino acids among Na,K β_1-, β_2-, and β_3-subunits or H,K β-subunits. Significantly, however, in all β-subunits the positions of some positively charged and hydrophobic amino acids are conserved in this region. In this study we have constructed a variety of β-subunit mutants by individually mutating several hydrophilic and hydrophobic residues in the C-terminus of the *Xenopus* β_3-subunit. Expression of the cRNAs in *Xenopus* oocytes allowed us to analyze the ability of these β-subunit mutants to assemble with exogenous *Xenopus* α-subunits and to form functional Na,K pumps at the plasma membrane. Our data support the

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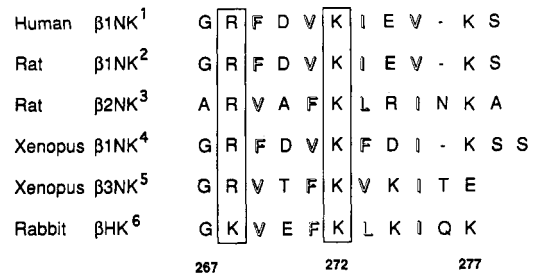

FIGURE 1: Sequence comparison of the C-termini of Na,K-ATPase (NK) and H,K-ATPase (HK) β-subunits. Conserved positions of positively charged amino acids are bordered. Hydrophobic amino acids are outlined. [1]Kawakami et al. (1986). [2]Young et al. (1987). [3]Vasallo et al. (1989). [4]Verrey et al. (1989). [5]Good et al. (1990). [6]Reuben et al. (1990). Amino acids are given in the one-letter code. The numbers refer to the position of amino acids in the *Xenopus* β_3-subunit.

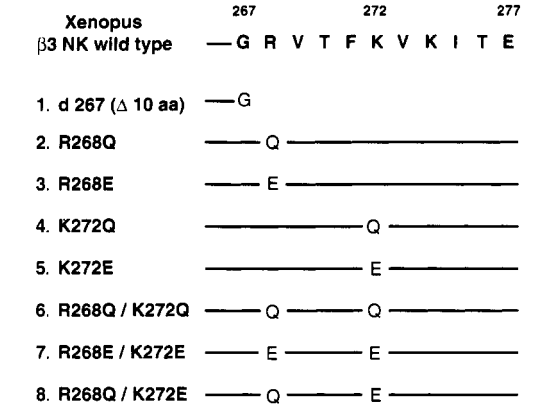

FIGURE 2: Deletion and point mutations in the C-terminus of the *Xenopus* β_3-subunit. The complete amino acid sequence of the last 11 amino acids of the wild-type β_3-subunit is given at the top. Diagrams 1–8 represent the deletion mutant (1) and the mutants with point mutations in the positively charged amino acids (2–8). Indicated are the amino acid changes introduced into the wild-type β_3-subunit.

hypothesis that the C-terminus of the β-subunit forms an amphipathic β-strand and that a hydrophobic domain is required for its interaction with the α-subunits.

MATERIALS AND METHODS

Site-Directed Mutagenesis. Figure 1 shows a comparison of the sequence of the last 10 amino acids of the C-termini of different Na,K β- and H,K β-subunits. By using site-directed mutagenesis, we replaced (1) the positively charged Arg (R268) and/or Lys (K272) in *Xenopus* β_3-subunits by the negatively charged Glu (E) and/or the polar Gln (Q), (2) the hydrophobic Val (V269), Phe (F271), Val (V273), or Ile (I275) by the polar Asn (N), and (3) V269 and F271 and/or V273 and I275 by the less hydrophobic Ala.

The deletion mutant d267 and the single and multiple point mutations (Figures 2 and 8) were introduced into the cDNA of the *Xenopus* β_3-subunit according to the polymerase chain reaction (PCR) method of Nelson and Long (1989). Mutagenic deoxynucleotides were synthesized with a DNA synthesizer (Applied Biosystems, PCR-Mate) according to the instructions of the manufacturer. In a first step, a 517–528-bp DNA fragment was amplified (20 temperature cycles: 94 °C for 1 min, 40 °C for 1 min, and 72 °C for 1 min) between the sense mutation-directing primer (22–29 bases in length for single mutations and 37–41 bases in length for double mutations) and the inverse hybrid primer [β_3 A1325–T1342 + 20 nucleotides corresponding to primer D of Nelson and Long (1989)] using 50 ng/100 μL of each primer, 10 ng of the linearized plasmid pSD5β_3 (Good et al., 1990) as a template, 200 μM each dNTP (Pharmacia), and 2.5 units of Taq polymerase (AmpliTaq polymerase, Perkin-Elmer Cetus) in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatin.

In a second step, the inverse DNA strands obtained by the first PCR were elongated by using 10 ng of the linearized template DNA (3 temperature cycles: 94 °C for 1 min, 40 °C for 1 min, and 72 °C 4 min).

In the last step, the mutated DNA is selectively amplified by adding 500 ng of the antisense primer D, 500 ng of sense primer β_3 C748–G767, and 1.25 units of Taq polymerase to the PCR tube (25 temperature cycles: 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min). Finally, the *Bam*HI (797) and *Hin*dIII (1317) restriction sites are used to introduce the mutated DNA fragment into the pSD5β_3 wild-type cDNA. To prepare mutant 4 (Figure 8), the mutant V269A/F271A was used as a template. The PCR-generated fragments were confirmed in all mutants by dideoxy sequencing (Sanger et al., 1977). No unintended mutations were found.

Microinjection of Xenopus Oocytes. Stage V–VI oocytes were obtained from *Xenopus* females (Noordhoek, Republic of South Africa) by removal of ovary segments from anesthetized frogs as described (Geering et al., 1989). After collagenase treatment and overnight incubation, oocytes were injected with *Xenopus* α_1cRNA alone or together with β_3 wild type or β_3 mutant cRNAs at concentrations indicated in the figure legends. cRNAs were obtained by *in vitro* transcription of linearized cDNA templates with SP6 RNA polymerase according to Melton et al. (1984). For templates, cDNAs from α_1 cloned from *Xenopus* kidney-derived A_6 cells (Verrey et al., 1987), from β_3 cloned from a *Xenopus* neurula library (Good et al., 1990), or from β_3 mutants were subcloned into the plasmid pSD5, which allows for synthesis of capped, full-length, poly(A)$^+$ RNA (Good et al., 1988).

Metabolic Labeling of Oocytes and Immunoprecipitation of α- and β-Subunits. Oocytes injected with cRNAs were incubated in modified Barth's solution (MBS) containing 1.5–2.5 mCi/mL ^{35}S-methionine for 4–48 h at 19 °C. Oocytes were then subjected to different chase periods in MBS containing 10 mM cold methionine as indicated in the figure legends. After the pulse–chase period, Triton extracts were prepared and the α- and β_3-subunits and mutants were immunoprecipitated as described (Jaunin et al., 1992) with an anti-α (Ackermann & Geering, 1990) or an anti-β_3 serum (Good et al., 1990). Preliminary experiments showed that all mutants were translated from cRNA with similar efficiency in a reticulocyte lysate and show similar immunoreactivity to the β_3 wild type (data not shown). In preliminary experiments, we tested the amount of cRNA of mutants to be injected into oocytes in order to give similar or slight overexpression of the mutants comparted to β_3 wild type. This precaution was taken to avoid mutants becoming limiting for α–β complex formation due to underexpression.

^{3}H-Ouabain Binding and ^{86}Rb Flux Measurements. The number and transport activity of Na,K pumps expressed at the plasma membrane of oocytes was assessed by ^{3}H-ouabain binding and ^{86}RbCl flux measurements essentially as described (Jaunin et al., 1992). In brief, oocytes were loaded with sodium for 1 h at room temperature with a potassium-free solution (110 mM NaCl and 10 mM Tris-HCl, pH 7.4). Ten to 15 oocytes were then incubated with 1 μM [21,22-^{3}H]ouabain (Amersham, specific activity 36 Ci/mmol) in solution 2 containing 90 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and

10 mM Hepes (pH 7.4) for 12 min. After extensive washing, oocytes were individually solubilized in 100 μL of SDS and counted in 2 mL of Scintillator 299 (Packard). Nonspecific binding, measured in the presence of a 1000-fold excess of cold ouabain, amounted to 3–5% of total binding. All experimental data shown represent total binding. On other oocytes from the same batch, ^{86}Rb uptake was measured after one wash in solution 2 supplemented with 5 mM $BaCl_2$ to inhibit nonspecific rubidium uptake through K^+ channels. ^{86}Rb uptake was measured after incubation of oocytes for 12 min at room temperature in solution 2 containing 5 mM $BaCl_2$ and 5 mM $^{86}RbCl$ (1 mCi/mmol, $^{86}RbCl$ from Amersham, specific activity 1–8 mCi/mg of rubidium). After extensive washing, oocytes were individually counted as described above. Nonspecific ^{86}Rb uptake was determined by including 1 mM ouabain in the reaction mixture and amounted to 5–10% of total ^{86}Rb uptake. All experimental data shown represent total ^{86}Rb uptake.

Secondary Structure Prediction and Database Searches. The secondary structure of each sequence shown in Figure 1 was predicted with the algorithm of Ptitsyn and Finkelstein (1983), which considers a model of "floating logs" (determination of amphipathic structures) for a protein chain, as well as middle- and long-range interactions.

Using the UWGCG sequence analysis package (Devereaux et al., 1984), we constructed a consensus sequence and a profile matrix (program PROFILEMAKE) based on the 11–12 C-terminal residues of the ATPase β chains as shown in Figure 1. This profile was then used to search (program PROFILESEARCH) the NRL-3D database (release 12.0) of sequences with known 3D structures (Brookhaven Protein Data Bank, release 62). The corresponding pairwise alignments were generated with the program PROFILESEGMENT. A database of secondary structure elements was built from the Brookhaven Protein Data Bank, based on the HELIX and STRAND records of each entry. This database was then searched with each individual sequence shown in Figure 1 using the FASTA (Pearson & Lipman, 1988) program.

RESULTS

Mutations of the Conserved Positively Charged Amino Acids in the C-Terminus of the β-Subunit Do Not Affect Subunit Assembly. Deletions of 11 amino acids in the C-terminus of the mouse Na,K-ATPase β-chain have been shown to affect α–β subunit assembly (Renaud & Fambrough, 1991). To confirm these findings, we constructed a mutant of the *Xenopus* β_3-subunit in which the last 10 amino acids of its C-terminus were deleted (Figure 3, mutant 1). In an attempt to identify the amino acids in this region involved in the α–β interaction, we mutated the positively charged amino acids arginine 268 (R268) and lysine 272 (K272) into glutamine (Q) or glutamic acid (E) (Figure 2, mutants 2–8).

These β-subunit mutants were expressed in *Xenopus* oocytes and their ability to assemble with α-subunits was tested by following the stabilization of coexpressed α-subunits and the glycosylation processing of the β-subunits in oocytes subjected to a pulse–chase protocol.

As previously described (Jaunin et al., 1992) and illustrated in Figure 3, assembly of β_3-subunits with α-subunits leads to an increased cellular accumulation of the α-subunit in oocytes compared to cells expressing α-subunits alone (Figure 3A, compare lanes 1 and 2). In addition, β_3-subunits coexpressed with α-subunits mainly accumulate in their fully glycosylated form, indicating that the α–β complexes have left the ER

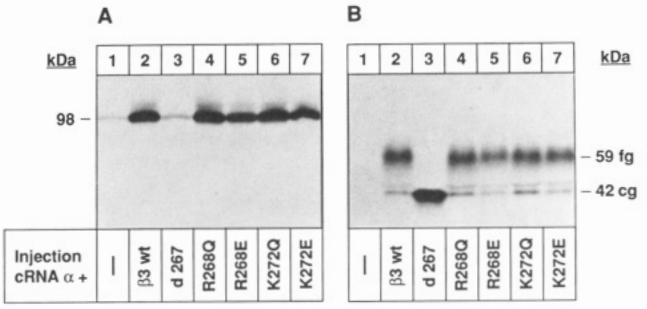

FIGURE 3: Cellular accumulation of α-subunits and glycosylation processing of β-subunits in oocytes expressing mutants affected in a single positively charged amino acid of the C-terminus of β_3-subunits. Oocytes were injected with 5 ng of α_1 cRNA alone or together with 0.2 ng of β_3 wild-type ($\beta_3$wt) or mutant cRNA, labeled for 5 h with ^{35}S-methionine (2.5 mCi/mL) and subjected to a 21-h chase period. Triton extracts were prepared and α- and β-subunits were immunoprecipitated from aliquots containing 10^6 cpm as described in Materials and Methods. (A) Immunoprecipitations of α-subunits. (B) Immunoprecipitations of β-subunits. Shown are the fully glycosylated (fg) β-forms with a molecular mass of 59 kDa and the core glycosylated (cg) β-forms with a molecular mass of 42 kDa. For description of the mutants see Figure 2. One out of three similar experiments is shown.

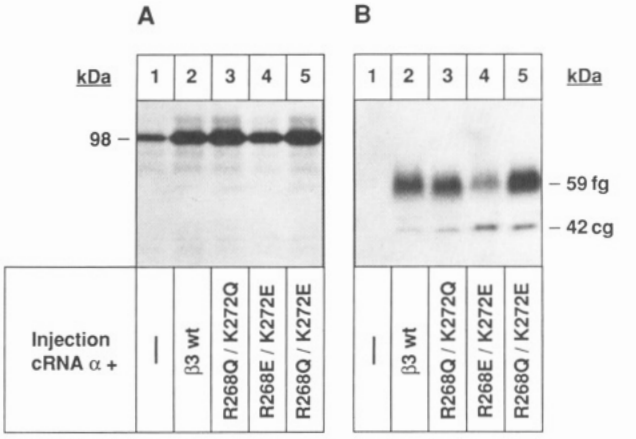

FIGURE 4: Cellular accumulation of α-subunits and glycosylation processing of β-subunits in oocytes expressing mutants affected in two positively charged amino acids of the C-terminus of β_3-subunits. For details see Figure 3.

(Figure 3B, lane 2). α-Subunits coexpressed with the deletion mutant (d267) are not stabilized and the amount of the α-subunits detected is as low as in oocytes expressing α-subunits alone (Figure 3A, compare lanes 3 and 1). Furthermore, the deletion mutant itself remains in its core glycosylated form (Figure 3B, lane 3), indicating that it is retained in the ER in an unassembled state (Ackermann & Geering, 1990). As previously shown by Renaud and Fambrough (1991), these results suggest that deletion mutants of the β-subunit lacking 10 amino acids in the C-terminus are no longer able to associate with α-subunits.

All mutants with changes at R268 and K272 to either an uncharged (R268Q, K272Q) or a negatively charged (R268E, K272E) amino acid were able to stabilize coexpressed α-subunits (Figure 3A, lanes 4–7). In addition, they became fully glycosylated (Figure 3B, lanes 4–7). Similarly, double mutants in which both R268 and K272 were mutated (R268Q/K272Q, R268E/K272E, and R268Q/K272E) also increased the cellular accumulation of α-subunits as compared to oocytes expressing α-subunits alone (Figure 4A, compare lane 1 to lanes 3–5). A small reduction in the efficiency of subunit assembly was only evident with the double mutant which contains two negatively instead of positively charged amino

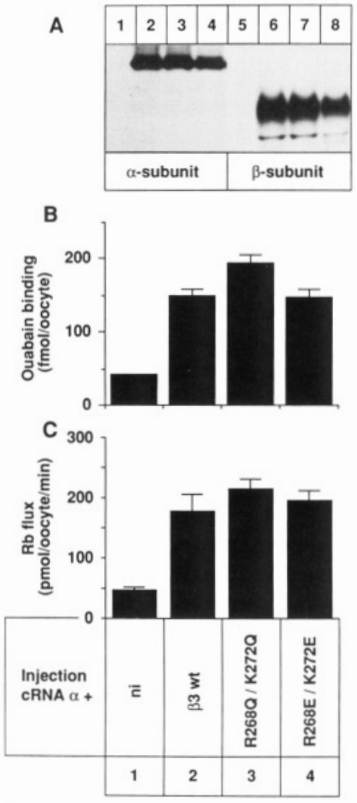

FIGURE 5: Functional expression of Na,K pumps in oocytes expressing mutants affected in positively charged amino acids of the C-terminus of β_3-subunits. Oocytes from one animal were either not injected (ni) or injected with 5 ng of α cRNA together with 0.3 ng of β_3 wild type ($\beta_3$wt) or β_3 mutant cRNA. (A) Immunoprecipitations of α- and β-subunits from oocytes that were not injected (lanes 1 and 5), injected with α cRNA and $\beta_3$wt (lanes 2 and 6), R268Q/K272Q (lanes 3 and 7), or R268E/K272E (lanes 4 and 8) cRNA. One batch of oocytes was labeled with ^{35}S-methionine (1.5 mCi/mL) for 21 h followed by a chase period of 4 days. α- (lanes 1–4) and β-subunits (lanes 5–8) were immunoprecipitated from aliquots of Triton extracts containing 1.5×10^6 cpm as described in Materials and Methods. (B) ^{3}H-Ouabain binding to intact oocytes. A second batch of oocytes was incubated for 5 days and ^{3}H-ouabain binding was measured as described (Jaunin et al., 1992). Shown are the means ± SE, $n = 10$. (C) ^{86}Rb uptake into oocytes after 5 days of incubation. ^{86}Rb flux measurements were done on intact oocytes as described (Jaunin et al., 1992). Shown are means ± SE, $n = 10$.

acids (R268E/K272E). As compared to $\beta_3$wt (Figure 4, lanes 2), this mutant produced a slight reduction in the cellular accumulation of the α-subunit (Figure 4A, lane 4), as well as in the ratio between fully glycosylated and core glycosylated β-subunits (Figure 4B, lane 4).

To test whether α–β mutant complexes could form functional Na,K pumps at the plasma membrane after expressing the double mutants together with α-subunits in *Xenopus* oocytes, the number of α–β complexes at the cell surface was determined by measuring ^{3}H-ouabain binding and the Na,K pump activity by ^{86}Rb uptake into oocytes. The $\beta_3$wt coexpressed with exogenous α-subunits increased the number and the activity of Na,K pumps at the oocyte cell surface in parallel and by 3–4-fold over the level of noninjected oocytes (Figure 5B,C, compare lane 1 with lane 2). Despite the slightly reduced expression of α-subunits obtained with the double mutant R268E/K272E (Figure 5A, lane 4), neither the number of α–β complexes (Figure 5B) nor the Na,K pump activity (Figure 5C) was significantly different in oocytes expressing the double mutants as compared to cells expressing the $\beta_3$wt.

Thus, the two highly conserved positively charged amino acids in the C-terminal region of the β-subunit are probably not directly involved in the interaction of the β-subunit with the α-subunit and might not be responsible for the lack of association observed with the deletion mutant.

Hydrophobic Amino Acids in the C-Terminus of the β-Subunit Are Involved in Assembly with the α-Subunit. In the C-terminus of all β-subunits identified so far, a characteristic distribution of hydrophilic amino acids and hydrophobic residues is observed (Figure 1). The algorithm of Ptitsyn and Finkelstein (1983) predicts a β-strand structure for this region (Figure 6A). In order to confirm this hypothesis, we searched the NRL-3D database with a computed consensus sequence derived from the six sequences shown in Figure 1. Figure 6B shows the six sequences most similar to the computed consensus with Z-scores between 2.68 and 5.41. Only one sequence with the third best Z-score shows an α-helical conformation (2FNR). In addition, each sequence shown in Figure 1 was used to search a database containing only sequences corresponding to defined secondary structure motifs. The 3–4 sequences most similar to each individual C-terminus of β-subunits shown in Figure 6A corresponded to a β-strand. A selection of the most similar sequences found during these searches are listed in Figure 6B (2CD4, 1REI, 2BPK, 1R08, and 1MLI).

When the C-terminal amino acids are aligned to form a β-strand-like secondary structure, all the hydrophobic amino acids are exposed on one side of the polypeptide, creating a continuous hydrophobic domain (Figure 7). To test the hypothesis that this hydrophobic domain could serve as an interaction site between the β- and the α-subunit, we mutated the four most C-terminal hydrophobic amino acids. Valine 269 (V296), phenylalanine 271 (F271), valine 273 (V273), and isoleucine 275 (I275) were changed to the polar uncharged asparagine (N) or the less hydrophobic alanine (A) (Figure 8).

The ability of the mutated β-chains to assemble with α-subunits was again determined in *Xenopus* oocytes by testing their stabilizing effect on coexpressed α-subunits and by following their glycosylation processing. Substitution of the hydrophobic amino acids with asparagine produced different effects on the efficiency of the α–β subunit assembly. Mutants V269N and I275N could efficiently associate with the α-subunit and led to an accumulation of the α-subunit which was similar to the one observed with $\beta_3$wt (Figure 9A, compare lane 3 with lane 4 and lane 7 with lane 9). Consistently, a lower accumulation of α-subunits was observed with the mutant V273N (Figure 9A, compare lane 7 with lane 8) and the most pronounced reduction was obtained with the mutant F271N (Figure 9A, compare lane 3 with lane 5) and the double mutant V269N/F271N (Figure 9A, lane 6).

To further assess the requirement for a hydrophobic domain in the C-terminus of the β-chain for α–β interaction, we achieved a progressive change in the hydrophobicity by introducing the less hydrophobic alanine. Significantly, the mutants V269A/F271A and V273A/I275A showed a decreased assembly efficiency as compared to $\beta_3$wt (Figure 9A, compare lane 10 with lanes 11 and 12). Finally, mutation of all four hydrophobic residues to alanine (mutant 4, Figure 8) abrogates the ability to associate with the α-subunit (Figure 9A, compared lane 2 with lane 13).

The lower efficiency or the inability of some of the described mutants to assemble with α-subunits is also reflected in the partial or complete ER retention of the mutated β-chains. The ratio between fully glycosylated and core glycosylated

			Secondary structure Ptitsyn & Finkelstein
A. Human	β1NK	GRFDVKIEV-KS	β–strand
Rat	β1NK	GRFDVKIEV-KS	β–strand
Rat	β2NK	ARVAFKIRINKA	β–strand
X. laevis	β1NK	GRFDVKFDI-KS	β–strand
X. laevis	β3NK	GRVTFKVKITE	β–strand
Rabbit	βHK	GKVEFKLKIQE	β–strand
Cons[a]		**GRLDIKIEITKS**	β–strand

				Z–score[b] Max=6.17	Secondary[c] structure	
B.	3PGM[d]	21 – 31	GWVDVKLSA-KG	5.41	β–strand	Phosphoglycerate mutase
	2BUS	47 – 57	GKINLKHRG-KC	3.69	β–strand	Proteinase inhibitor IIA bovine
	2FNR	277–287	DWIEYKRQL-KK	3.28	α–helix	Ferredoxin–NADP+ reductase
	2GD1	51 – 61	GRLDAEVSVNG	3.10	β–strand	Glyceraldehyde–3–phosphate dehydrogenase
	2DPV	531–541	GKLVFKAKL-RA	2.82	β–strand	Parvovirus VP2
	3FAB	209–219	TKVDKKVEP-KS	2.68	β–strand	Immunoglobulin heavy chain
	2CD4	166–174	KKVEFKIDIVVL	NA[e]	β–strand	T–cell surface glycoprotein CD4
	1REI	99 – 107	FGQGTKLQIT	NA[e]	β–strand	Bence–Jones Immunoglobulin light chain
	2BPK	351–359	GRFDRRVSVCA	NA[e]	β–strand	cAMP–dependent protein kinase
	1R08	122–133	VRFDSEYTILAT	NA[e]	β–strand	Rhinovirus 14 VP1
	1MLI	1 – 11	MLFHVKMTV-KL	NA[e]	β–strand	Muconolactone isomerase

FIGURE 6: Secondary structure prediction of the C-terminal domains of ATPase β-chains shown in Figure 1 (A) and the sequences with known 3D structure to which they are most similar (B). [a] The consensus sequence was deduced using the GCG package (Devereaux et al., 1984). [b] The five most similar sequences with β-strand structure are shown and listed according to their Z scores. [c] The secondary structure of the sequences as determined by X-ray crystallography. [d] The Brookhaven Protein Data Bank entry codes are given at the beginning of each line. [e] These sequences were found using FASTA (Pearson & Lipman, 1988) searches on a database containing experimentally determined secondary structure elements.

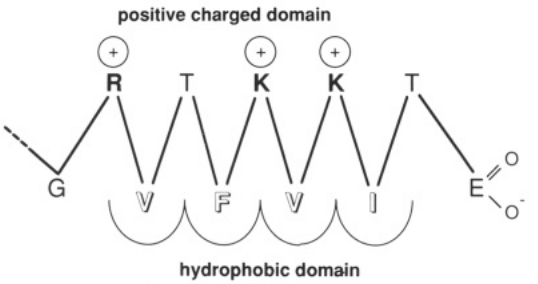

FIGURE 7: Hypothetical secondary structure of the C-terminus of *Xenopus* β_3-subunits. The 10 last amino acids are aligned in a β-strand-like structure delimiting a hydrophobic from a hydrophilic domain. For explanation see text.

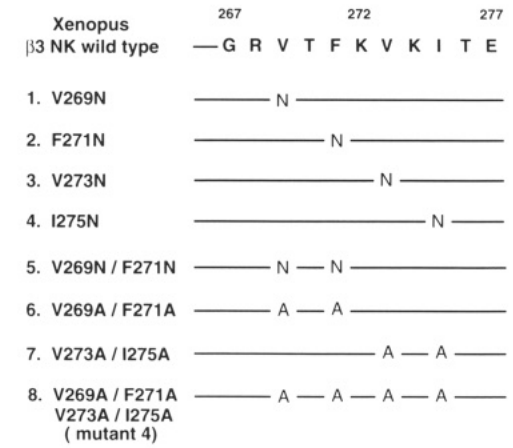

FIGURE 8: Point mutations in the hydrophobic amino acids of the C-terminus of the *Xenopus* β_3-subunit. Same representation as in Figure 2.

β-subunits was greatly decreased for V273N (Figure 9B, lane 8), V269A/F261A (Figure 9B, lane 11), and V273A/I275A (Figure 9B, lane 13). For F271N (Figure 9B, lane 5), V269N/F271N (Figure 9B, lane 6), and mutant 4 (Figure 9B, lane 13) all the proteins appear to be in their core glycosylated form confined to the ER.

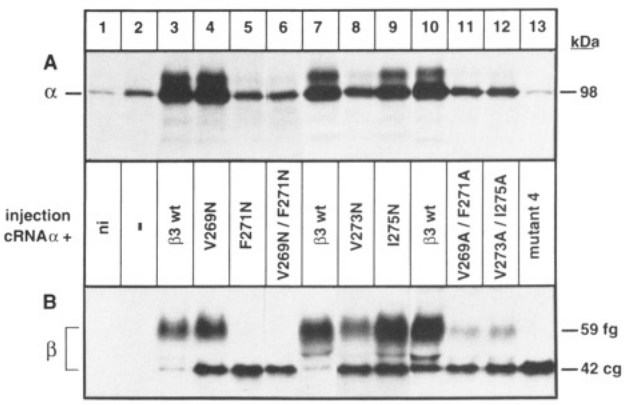

FIGURE 9: Cellular accumulation of α-subunits and glycosylation processing of β-subunits in oocytes expressing mutants affected in hydrophobic amino acids of the C-terminus of β_3-subunits. Oocytes were either not injected (ni) or injected with 5 ng of α cRNA alone or with 5 ng of α cRNA together with 0.3 ng of β_3 wild type ($\beta_3$wt) or 0.3–0.9 ng of β_3 mutant cRNA (see Materials and Methods). Oocytes were then labeled for 5 h with ^{35}S-methionine and subjected to a 24-h chase period. α-Subunits (A) and β-subunits (B) were immunoprecipitated from aliquots of Triton extracts containing 2×10^6 cpm as described in Materials and Methods. Symbols are as in Figure 3. For a description of the mutants see Figure 8. Data were obtained from three different batches of oocytes (lanes 1–6, 7–9, and 10–13) that were injected with different combinations of cRNAs. In all batches of oocytes, the expression of endogenous and exogenous α-subunits synthesized alone was similar to that shown in lane 1 and 2, respectively.

The functional expression of the mutant α–β complexes was tested by ouabain binding on intact oocytes. Compared to oocytes injected with αcRNA alone (Figure 10A, lanes 1 and 9), a similar increase (about 4–5-fold) in the number of Na,K pumps at the cell surface was achieved in oocytes expressing α–βwt (Figure 10A, lanes 1 and 10), α-V269N (Figure 10A, lane 3), α-V273N (Figure 10A, lane 11), or α-I275N (Figure 10A, lane 12) complexes. Oocytes expressing the mutant F271N show about a 2-fold increase of Na,K pumps at the cell surface (Figure 10A, compare lanes 1 and

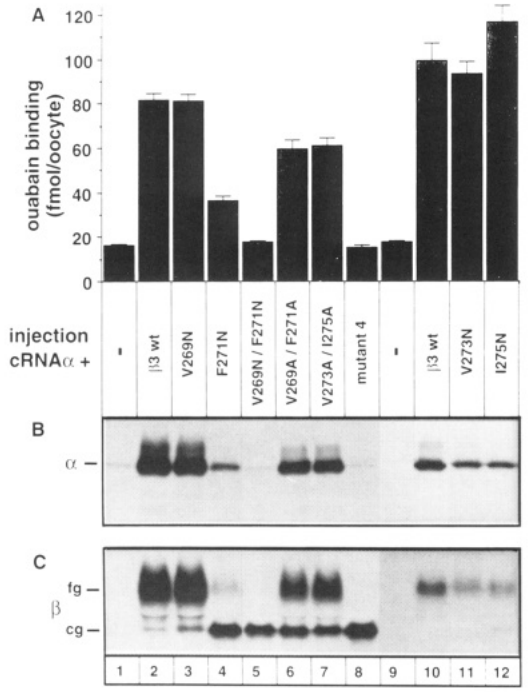

FIGURE 10: Functional expression of Na,K pumps in oocytes expressing mutants affected in hydrophobic amino acids of the C-terminus of β_3-subunits. (A) ^{3}H-Ouabain binding on intact oocytes. Oocytes of two different batches (lanes 1–8 and lanes 9–12) were injected with 5 ng of α cRNA alone or 5 ng of α cRNA together with 0.5 ng of β_3 wild type ($\beta_3$wt) or 0.5–1.5 ng of β_3 mutant cRNA (see Materials and Methods). Five days after incubation, ^{3}H-ouabain binding was performed as described in Materials and Methods. Shown are the means ± SE, n = 13–16. Unpaired Student's t-test: All values (lanes 2–4, 6, 7, and 10–12) are significantly different ($p < 0.001$) from the respective value of ouabain binding measured in the same batch of oocytes expressing α-subunits alone (lanes 1 and 9) except for the values obtained in oocytes expressing α-V269N/F271N (lane 5) or α-mutant 4 (lane 8) complexes ($p > 0.05$). Compared to oocytes expressing α–$\beta_3$wt complexes (lane 2), a significant decrease ($p < 0.001$) in the number of Na,K pumps was observed in the same batch of oocytes expressing α-F271N (lane 4), α-V269A/F271A (lane 6), or α-V273A/I275A (lane 7). (B) Immunoprecipitations of α-subunits. (C) Immunoprecipitation of β-subunits. For immunoprecipitation of α- and β-subunits, part of the oocytes injected for ouabain binding were labeled for 24 h with ^{35}S-methionine and subjected to a chase for 4 days. Thereafter, Triton extracts were prepared and α-subunits (B) and β-subunits (C) were immunoprecipitated from aliquots of Triton extracts containing 2×10^6 cpm (lanes 1–8) or 1×10^6 cpm (lanes 9–12). Exposure times of autoradiograms of the experiment shown in lanes 1–8 was 1 day for α-subunits and 4 days for β-subunits. For the experiment shown in lanes 9–12, the exposure times were 12 and 60 h for α- and β-subunits, respectively.

4) despite the low assembly efficiency demonstrated in the pulse–chase experiment shown in Figure 9. On the other hand, oocytes expressing the double mutant V269N/F271N have no more Na,K pumps than oocytes expressing α-subunits alone (Figure 10A, compare lanes 1 and 5). Compared to oocytes expressing α–βwt complexes, a significant reduction of Na,K pumps of about 25% is observed in oocytes expressing α-subunits together with the mutants V269A/F271A (Figure 10A, compare lane 2 with lane 6) or V273A/I275A (Figure 10A, compare lane 2 with lane 7). Significantly, the number of Na,K pumps falls to the level of oocytes expressing α-subunits alone when mutant 4 is coexpressed with α-subunits (Figure 10A, compare lane 1 with lane 8). The different expression of Na,K pumps observed with the various mutants was paralleled by the Na,K pump activity using ^{86}Rb uptake measurements (data not shown).

The number of Na,K pumps at the cell surface assessed by ouabain binding 5 days after cRNA injection reflects, on the one hand, the pattern of cellular accumulation of the α-subunits and, on the other hand, the proportion of core versus fully glycosylated β-subunits measured after a 4-day chase period (Figure 10B,C). Thus the mutants that yielded a low increase (F271N, V269A/F271A, and V273A/I275A) or no increase (V269N/F271N and mutant 4) in Na,K pump expression also caused a low accumulation (Figure 10B, lanes 4, 6, and 7) or no accumulation (Figure 10B, lanes 5 and 8) of α-subunits. Finally, these mutants remained partially (Figure 10C, lanes 4, 6 and 7) or entirely (Figure 10C, lanes 5 and 8) in their core glycosylated form, consistent with their partial or entire ER retention in an unassembled form. The decreased amount of core glycosylated β-species after a 4-day chase (Figure 10C) compared to a 24-h chase (Figure 9B) is due to the selective degradation of the unassembled high-mannose forms in the ER during the longer chase (Ackermann & Geering, 1990). Despite its lower assembly efficiency (Figure 9, lane 8, and Figure 10B,C, lane 11), the mutant V273N yielded a similar number of α–β complexes at the cell surface than $\beta_3$wt (Figure 10A, lane 11). This fact supports the observation made by Schmalzing et al. (1989) that the expression of functional Na,K pumps at the cell surface is controlled in oocytes and that only a limited number of α–β complexes reach the plasma membrane. As a consequence, β-mutants with only a slightly reduced assembly efficiency will yield similar numbers of Na,K pumps at the cell surface as βwt in the case where the mutant is slightly overexpressed compared to $\beta_3$wt.

Because of the lack of an antibody able to coprecipitate α–β complexes, we have in this study probed the assembly competence of β-subunits by an indirect test, namely, by following the ability of β-subunits to stabilize α-subunits. With this test we cannot exclude that the mutant V269N/F271N or the mutant 4 are indeed able to associate with the α-subunit but cannot stabilize it. To verify this possibility we coexpressed in a last series of experiments βwt and V269N/F271N mutants in oocytes together with α-subunits and assessed whether β-mutants were able to compete with βwt in the formation of α–β complexes. If the β mutants were indeed able to efficiently associate with α-subunits, we would expect that in the presence of βwt, increasing amounts of β mutants in excess over βwt would inhibit the formation of stable α–β wild-type complexes, resulting in a decreased cellular accumulation of α-subunits.

Figure 11 shows again that βwt but not β mutant V269N/F271N was able to stabilize coexpressed α-subunits (Figure 11A, lanes 2–4) and only βwt became fully glycosylated while the β mutant remained in the core glycosylated ER form (Figure 11B, lanes 3 and 4). Coinjection of βwt cRNA with increasing amounts of β mutant cRNA led to a progressive increase of the core glycosylated β mutant (Figure 11B, lanes 5–8, βm). This situation did not significantly influence either the amount of fully glycosylated βwt (Figure 11B, lanes 5–7, βwt, fg) or the amount of stabilized α-subunits (Figure 11A, lanes 5–7), at least in oocytes that expressed up to a 30-fold excess β mutant over βwt. Only a 100-fold excess of β mutant led to a decrease in the amount of accumulated α-subunits by about 50% (Figure 11A, lane 8) concomitant with a decrease in the amount of fully glycosylated βwt (Figure 11B, lane 8). The decrease in stable α–βwt complexes under this condition might not be due to a saturation of the oocyte's protein synthesis capacity since injection of similar amounts of βwt cRNA led to a higher accumulation of α-subunits as comapred to injection

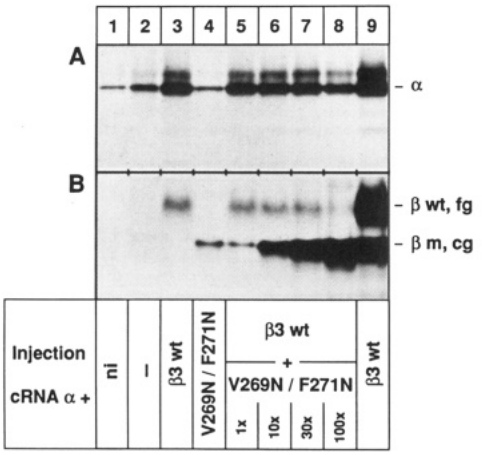

FIGURE 11: Competition of V269N/F271N mutant with βwt for assembly with α-subunits. Oocytes were injected as follows: lane 1, not injected; lane 2, 0.8 ng of α cRNA; lane 3, 0.8 ng of α cRNA plus 0.05 ng of $\beta_3$wt cRNA; lane 4, 0.08 ng of α cRNA plus 0.05 ng of mutant cRNA; lanes 5–8, 0.8 ng of α cRNA plus 0.05 ng of $\beta_3$wt cRNA plus 0.05 ng (lane 5, 1×), 0.5 ng (lane 6, 10×), 1.65 ng (lane 7, 30×), or 5 ng (lane 8, 100×) of mutant cRNA; lane 9, 0.8 ng of α cRNA plus 5 ng of $\beta_3$wt cRNA. After a 4-h pulse with ^{35}S-methionine and a 24-h chase, α-subunits (A) and β-subunits (B) were immunoprecipitated from Triton extracts. βwt, fg = fully glycosylated β_3 wild type; βm, cg = core glycosylated β-V269N/F271N mutant.

of β mutant cRNA (Figure 11A, compare lane 9 with lane 8).

Thus these data could indicate that the β mutant V269N/F271N at high concentrations either interferes in some noncompetitive way with the formation of stable α–βwt complexes or has some ability to associate with α-subunits forming unstable α–β complexes. However, the association affinity with the α-subunit of these mutants seems to be very low. This suggests that the C-terminus of the β-subunit is implicated not only in the stabilization of the α-subunit but also in the process of α–β subunit assembly itself.

DISCUSSION

Assembly of β-subunits with newly synthesized α-subunits is fundamental for the expression of functional Na,K-ATPase at the cell surface [for review see Geering (1991)]. In this study, we have used site-directed mutagenesis to identify the amino acid sequences in the ectodomain of the β-chain which are implicated in α–β subunit oligomerization. Our results indicate that a set of hydrophobic amino acids conserved in the C-terminus of different β-subunits of P-type ATPases are crucially involved in α–β subunit assembly.

Our results are in agreement with previous findings suggesting that the ectodomain of the β-chain is important for its interaction with the α-subunit (Geering et al., 1993; Omari et al., 1991; Renaud et al., 1992). Based on the observation that deletion of not more than 11 amino acids from the C-terminus of the β-subunit is sufficient to abolish the association capacity (Renaud & Fambrough, 1991), we attempted to identify the specific amino acids in this region of the β-subunit that are important for association with α-subunits.

The sequence of the C-terminal portion of different β-subunits shows a binary pattern of hydrophilic/charged and hydrophobic residues. This motif, when folded as a β-strand, forms an amphipathic secondary structure element. Secondary structure predictions and similarity searches with experimentally determined secondary structure elements showed that the C-terminal domain of each ATPase β-subunit (Figure 1) may indeed fold as a β-strand. This allowed us to identify in the C-terminus of the β-subunit two potentially important domains, one hydrophilic and positively charged and the other hydrophobic (Figure 7). Our data show that hydrophilic/charged amino acids do not significantly contribute to the α–β association. On the other hand, our results suggest that the hydrophobic domain composed of four amino acids contributes to the binding surface of the β-chain with the α-subunit. In particular, mutations of each of the four hydrophobic amino acids in the C-terminus of the β-subunit have a more or less pronounced effect on the α–β association efficiency. In addition, these residues appear to participate in a cooperative way since (1) a double mutation of V269 and F271 (mutant V269N/F271N) completely abolishes the assembly competence of the single mutants V269N and F271N and (2) replacement of two out of the four hydrophobic residues by the less hydrophobic alanine decreases the assembly efficiency, whereas replacement of all four amino acids by alanine completely abolishes it.

However, our present results cannot demonstrate whether the hydrophobic portion in the C-terminus of the β-subunit is sufficient to mediate oligomerization or whether other domains exist that are needed for a correct assembly of α- and β-subunits. Recent data obtained in our laboratory indeed indicate that chimeric proteins in which the transmembrane domain of the β-subunit of Na,K-ATPase is replaced by the transferrin receptor associate very inefficiently with α-subunits (Jaunin et al., 1994). In addition, a study that analyzed chimeric proteins between type II glycoproteins (similar to β-subunits of Na,K-ATPase) showed that the cytoplasmic and membrane anchor domain affect in a so far ill-defined way the subunit assembly of the ectodomains (Kundu et al., 1991).

The results obtained in this study from competition experiments show that at high levels of expression the V269N/F271N mutant is able to decrease the amount of stabilized α–βwt complexes. A similar observation was made by Omori et al. (1991), who reported that, in transfected cells expressing assembly-incompetent C-terminal deletion mutants of the β-subunit, the degree of assembly of the endogenous Na,K-ATPase subunits was decreased compared to untransfected cells. As suggested by Omori et al. (1991), these data could indicate that the β-mutants can interact with α- and/or βwt-subunits, even if affinity is low, and inhibit the α–β assembly. Alternatively, the mutants could associate with some limiting factor necessary for assembly. Our results could also indicate that β-subunits possess different domains which cooperate in the assembly process. It is indeed possible that the transmembrane and/or other domains may play a role in the initial steps of the assembly process to bring the two subunits together and permit thereby a stable interaction of the ectodomains. Alternatively, it is conceivable that the formation of stable α–β complexes is only possible after initial assembly of their ectodomains followed by the interaction among the transmembrane and/or other subunit domains.

Finally, the question arises what is the extracellular association site(s) in the α-subunit that could interact with the hydrophobic amino acids of the β-subunits. It has recently been shown that chimeric proteins between Ca^{2+}- and Na,K-ATPase that contain 161 amino acids of the C-terminus of Na,K-ATPase assemble with β-subunits (Lemas et al., 1992). Since Na,K-ATPase α-subunits from different species can associate with β-subunits from other species (Takeyasu et al., 1987; Schmalzing et al., 1992; Horowitz et al., 1990) as well as with β-subunits from H,K-ATPase (Eakle et al., 1992;

Horisberger et al., 1991; Noguchi et al., 1992), an evolutionarily conserved hydrophobic region that is important for assembly probably exists in the carboxyl terminus of the α-subunit. The precise location and the nature of this (these) interaction site(s) remains to be determined.

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